Megakaryocytes Regulate Expression of Pyk2 Isoforms and Caspase-mediated Cleavage of Actin in Osteoblasts*

Melissa A. Kacena[‡1], **Pierre P. Eleniste**[§], **Ying-Hua Cheng**[‡], **Su Huang**[§], **Mahesh Shivanna**[§], **Tomas E. Meijome**[‡], **Lindsey D. Mayo**[¶], **and Angela Bruzzaniti**[§2]

From the ‡Department of Orthopaedic Surgery and the ¶Herman B. Wells Center for Pediatric Research, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine and the §Department of Oral Biology, Indiana University School of Dentistry, Indianapolis, Indiana 46202

Background: Osteoblast proliferation and differentiation are critical for bone formation.
Results: Megakaryocytes increase osteoblast number and BrdU incorporation and induce cytoskeletal remodeling and the differential expression of Pyk2 isoforms in osteoblasts.
Conclusion: Megakaryocytes stimulate osteoblast proliferation and alter the ratio of alternatively spliced Pyk2 isoforms.
Significance: Pyk2 may be an important target for treatments aimed at increasing skeletal bone formation.

The proliferation and differentiation of osteoblast (OB) precursors are essential for elaborating the bone-forming activity of mature OBs. However, the mechanisms regulating OB proliferation and function are largely unknown. We reported that OB proliferation is enhanced by megakaryocytes (MKs) via a process that is regulated in part by integrin signaling. The tyrosine kinase Pyk2 has been shown to regulate cell proliferation and survival in a variety of cells. Pyk2 is also activated by integrin signaling and regulates actin remodeling in bone-resorbing osteoclasts. In this study, we examined the role of Pyk2 and actin in the MK-mediated increase in OB proliferation. Calvarial OBs were cultured in the presence of MKs for various times, and Pyk2 signaling cascades in OBs were examined by Western blotting, subcellular fractionation, and microscopy. We found that MKs regulate the temporal expression of Pyk2 and its subcellular localization. We also found that MKs regulate the expression of two alternatively spliced isoforms of Pyk2 in OBs, which may regulate OB differentiation and proliferation. MKs also induced cytoskeletal reorganization in OBs, which was associated with the caspase-mediated cleavage of actin, an increase in focal adhesions, and the formation of apical membrane ruffles. Moreover, BrdU incorporation in MK-stimulated OBs was blocked by the actin-polymerizing agent, jasplakinolide. Collectively, our studies reveal that Pyk2 and actin play an important role in MK-regulated signaling cascades that control OB proliferation and

may be important for therapeutic interventions aimed at increasing bone formation in metabolic diseases of the skeleton.

Bone remodeling occurs through the combined actions of OBs[3] that synthesize new bone and osteoclasts that degrade bone. Within the bone marrow and stem cell niche, OB precursors are intimately associated with hematopoietically derived progenitors as well as with MKs. MKs are responsible for the production of thrombocytes, which are necessary for blood clotting. Although the mechanism has yet to be fully elucidated, a growing body of evidence also suggests that MKs play a key role in regulating skeletal homeostasis through direct and indirect effects on OBs and osteoclasts (1–6). We previously reported that OB cell number is significantly increased when OBs are co-cultured with MKs *in vitro* (1, 2, 4). In addition, our studies reveal that MKs stimulate OB proliferation in part through direct cell-cell contact and via a mechanism that requires integrin engagement (5). Furthermore, in GATA-1-deficient mice in which MK numbers are increased, bone mineral density is increased (6), and histological evaluation reveals an increase in OB numbers on trabecular surfaces, suggesting that MKs stimulate OB proliferation *in vivo*.

The focal adhesion tyrosine kinases Pyk2 and FAK are linked to the proliferation, migration, and activity of a variety of mesenchymal, epithelial, and hematopoietic cell types (7–10). Our group and others have reported that deletion of the *Pyk2* gene in mice leads to an increase in bone mass, which is due to defects in the activity of OBs and osteoclasts (7, 11). In addition, Pyk2 is activated by integrin engagement and is involved in F-actin reorganization in a variety of cells, including osteoclasts (8, 12). Recently, it was also established that both Pyk2 and FAK regulate cell proliferation and survival via a process that involves the degradation of the tumor suppressor protein, p53 (13, 14). In this study, we examined the role of Pyk2 and actin in

¹ To whom correspondence may be addressed: Dept. of Orthopaedic Surgery, Indiana University School of Medicine, 1120 South Dr., FH115, Indianapolis, IN 46202. Tel.: 317-278-3482; Fax: 317278-9568; E-mail: mkacena@iupui.edu.
² To whom correspondence may be addressed: Dept. of Oral Biology, Indiana University School of Dentistry, 1121 W. Michigan St., DS241, Indianapolis, IN 46202. Tel.: 317-278-3742; Fax: 317-278-1416; E-mail: abruzzan@iupui.edu.

³ The abbreviations used are: OB, osteoblast, MK, megakaryocyte; Osx, osterix; Ocn, osteocalcin; FAK, focal adhesion kinase; qPCR, quantitative real time PCR; PGE₂, prostaglandin E₂; AA, ascorbic acid; BrdU, bromodeoxyuridine.

the stimulatory effect of MKs on OB proliferation. We demonstrate that MKs increase OB cell number and bromodeoxyuridine (BrdU) incorporation in OBs. In addition, MKs regulate the expression of two distinct Pyk2 isoforms in OBs that are likely to be associated with OB differentiation and/or proliferation. Moreover, MKs stimulate the transient increase in the caspase-mediated cleavage of actin in OBs, which is independent of apoptosis and is important for cytoskeletal re-organization in OBs in the presence of MKs. Finally, we report that actin depolymerization plays an important role in the MK-stimulated increase in OB proliferation. Taken together, our findings suggest that MKs regulate cytoskeletal rearrangement and the isoform-specific expression of Pyk2 in OBs, which may be important for OB proliferation and function.

EXPERIMENTAL PROCEDURES

Preparation of Neonatal Calvarial Osteoblasts—Murine calvarial cells were prepared using a modification of a protocol described previously (15–18). Briefly, calvaria from 2- to 3-day neonatal mice were pretreated with 10 mM EDTA in phosphate-buffered saline (PBS) for 30 min. The calvaria were then subject to sequential collagenase digestions, and cells were collected. Fractions 3–5 were used as the starting population that consisted of ~95% OB or OB precursors, as determined by a variety of criteria (15, 17, 18). For experiments with MKs, OBs were seeded at 2×10^4 cells/ml in 6-well plates or in 10-cm^2 dishes. All mice used for the generation of OBs were handled according to an approved IACUC animal protocol.

Preparation of Fetal Liver-derived MKs—Murine MKs were prepared as described previously (6, 19). In brief, fetuses were dissected from pregnant mice at day E13–15. All mice for the preparation of MKs were handled according to an approved IACUC animal protocol. The livers were removed, and single cell suspensions were made by forcing cells through sequentially smaller gauge needles (18, 20, and 23 gauge). Cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) + 10% FCS and then seeded in 100-ml culture dishes (five fetal livers/100-mm dish), in DMEM + 10% FCS containing 1% murine thrombopoietin. After 3–5 days, cells became confluent, and MKs were obtained by separating them from lymphocytes and other cells using a one-step albumin gradient. The bottom layer was 3% albumin in PBS (bovine albumin, protease-free and fatty acid-poor, Serological Proteins Inc., Kankakee, IL); the middle layer was 1.5% albumin in PBS, and the top layer was media containing the cells to be separated. All of the cells were sedimented through the layers at $1 \times g$ for ~40 min at room temperature. The MK-rich fraction was collected from the bottom of the tube, and MKs were seeded at 1×10^5 cells/well in 6-well plates and 2×10^5 cells/dish in 10-cm dishes.

OB-MK Co-cultures—For all experiments, OBs were cultured alone for 4 days, and MKs were added for various times as indicated. MKs, which are nonadherent cells, were then removed from OBs by thorough washing as described previously, and purified OBs were subsequently analyzed (4, 6). Briefly, MKs were removed from OBs by repeated washing (four washes) with ice-cold PBS, which results in greater than 90% purity of OB lineage cells. All cultures, including control

OBs not exposed to MKs, were similarly washed to control for potential wash-related signaling effects on OBs. Furthermore, prior to harvesting OBs, microscopic examination of cell cultures was performed to ensure that only OBs remained in culture wells.

BrdU Incorporation—OBs were cultured alone or co-cultured with MKs for 4 days prior to analysis using the BrdU cell proliferation assay kit (Cell Signaling Technology Inc., Danvers, MA). In brief, 10 μM BrdU was added to the cultures 16 h prior to assay. Cells were fixed; DNA was denatured, and BrdU mouse mAb was added, followed by an anti-mouse IgG HRP-linked antibody and finally the HRP substrate that recognizes the bound antibody. The magnitude of absorbance at 450 nm is proportional to the BrdU incorporated into the cells. In studies examining the short term effects of MKs on the OB cytoskeleton using the jasplakinolide, OBs were cultured for 4 days, and MKs were added to OBs 6 h prior to terminating the experiment. BrdU (10 μM) with or without jasplakinolide (1 μM) was added for the final 2 h of the co-culture. MKs were removed by washing, and OBs were processed for absorbance.

Western Blotting—OB cultures were rinsed with ice-cold PBS and lysed in modified RIPA (mRIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1% aprotinin, and 0.1 mM Na_3VO_4). Primary monoclonal antibodies for Western blotting were all from BD Biosciences. Western blotting was performed using the enhanced chemiluminescence detection assay. X-ray films were scanned, and images were processed with Adobe Photoshop 5.0. As necessary, protein bands were quantified by densitometric analysis using ImageJ software.

Analysis of Pyk2-interacting Proteins—Proteins interacting with Pyk2 were identified by the Proteomics Core at Indiana University Department of Biochemistry. Briefly, Pyk2-GST was expressed in bacteria and purified. Cell lysates were prepared from OBs cultured alone or in the presence of MKs and separated on the Pyk2-GST column. Bound proteins were eluted and resolved by SDS-PAGE. Proteins were subject to in-gel digestion and analyzed using the Thermo-Fisher Scientific LTQ and Waters nano-UPLC system. Data base search was performed using both Sequest and X!Tandem algorithms.

Subcellular Fractionation—OB subcellular fractionation was performed using the Proteo-Extract kit from Calbiochem. Cell pellets were sequentially resuspended in different extraction buffers to generate a cytosolic protein extract (fraction 1), a membrane/organelle protein extract (fraction 2), a nucleic protein extract (fraction 3), and a cytoskeletal matrix protein extract (fraction 4).

RNA Isolation and Reverse Transcription-PCR—Total RNA was isolated from samples with the RNA mini prep kit (catalog no. 74105) from Qiagen. Total RNA was treated with DNase I (Ambion). cDNA was generated as described by the manufacturer (catalog no. 04379012001, Roche Applied Science) and used as the template for PCR (Thermo Scientific AB-0192/A) using Pyk2 primers spanning the mRNA isoform splice sites as follows: 5′-AGCAAGAAAGGAATGCTCGCTACC; 5′-TTC-CACCATCTGCTTCTGCTGTCT. GAPDH was used as an internal control as follows: 5′-AACTTTGGCATTGTG-GAAGG and 5′-CCCTGTTGCTGTAGCCGTAT. PCR frag-

ments were separated on 2.0% agarose gels, stained with ethidium bromide and imaged with a Bio-Rad Gel Doc XR system.

Quantitative Real Time PCR—OB cultures were washed four times with PBS to remove MKs, and OBs alone were used for quantitative real time PCR (qPCR) analysis. Total RNA was isolated from OBs using TRIzol reagent (Invitrogen). Total RNA from OBs was treated with DNase I (Qiagen) and used to generate cDNAs by reverse transcription according to the manufacturer's instructions (first strand cDNA synthesis kit; Roche Applied Science). qPCRs were performed in an MX3000 detection system using SYBR Green PCR reagents as described by the manufacturer (Stratagene). For each primer pair, a calibration curve was performed, and all oligonucleotides were tested to ensure specificity and sensitivity. For each OB sample, arbitrary units obtained using the standard curve and the expression of GAPDH were used to normalize the amount of the mRNA transcript. The following forward and reverse primer pairs were used to detect mRNA for c-*fos*, *runx2*, alkaline phosphatase (*alpl*), type I collagen (*col1a1*; *Col1*), osterix (*sp7*; Osx) and osteocalcin (*bglap*; Ocn): c-*fos*, 5′-ACTTCTTGTTTCCGGC and 5′-AGCTTCAGGGTAGGTG; *runx2*, 5′-CGACAGTCC-CAACTTCCTGT and 5′-CGGTAACCACAGTCCCATCT; *alpl*, 5′-GCTGATCATTCCCACGTTTT and 5′-CTGGGCC-TGGTAGTTGTTGT; *colla1*, 5′-CAGGGAAGCCTCTTTC-TCCT and 5′-ACGTCCTGGTGAAGTTGGTC; *sp7*, 5′-CCC-TTCTCAAGCACCAATGG and 5′AGGTGGGTAGTCATT-TGCATAG; *bgalp*, 5′-AGCAGGAGGGCAATAAGGT and 5′-TTTGTAGGCGGTCTTCAAGC; *gapdh*, 5′-CGTGGGG-CTGCCCAGAACAT and 5′-TCTCCAGGCGGCACGT-CAGA.

Caspase Activity Assay—OBs were isolated as above and seeded at 20,000 cells/well (optimal, pre-tested) into a black 96-well microtiter plate for quantitation of fluorescence intensity using a fluorescence plate reader (Molecular Devices SpectraMax M5, Sunnyvale, CA) or in a tissue culture grade 96-well plate to confirm findings via fluorescence microscopy. After 24 h, cells were serum-starved overnight. The next morning some cultures were treated with 20 μM Z-benzyloxycarbonyl-fluoromethyl ketone (Z-VAD-FMK) (Apotech, San Diego) for 16 h to inhibit caspase 3 activity. Following treatment, MKs were added to some cultures for 1 or 16 h, and then all cultures were examined for detection of caspase 3/7 activity using the Magic Red™ real time protease detection kit as described by the manufacturer (Immunochemistry Technologies, LLC, Bloomington, MN). Briefly, the target substrate is linked to a red fluorophore that can be cleaved by caspase 3/7. As caspase 3/7 activity progresses, more substrate is cleaved, and the red fluorescent signal also increases.

Apoptosis Assay—Apoptosis was assessed by examining the levels of annexin V using flow cytometry. Cells were washed once in DMEM followed by the addition of annexin V conjugated with allophycocyanin. Cells were incubated on ice for 15 min. Cells were washed and resuspended in DMEM followed by the addition of 10 μl of 10 ng/ml propidium iodide. Cells were incubated at room temperature for another 10 min, and data were collected on a FACSCalibur flow cytometer.
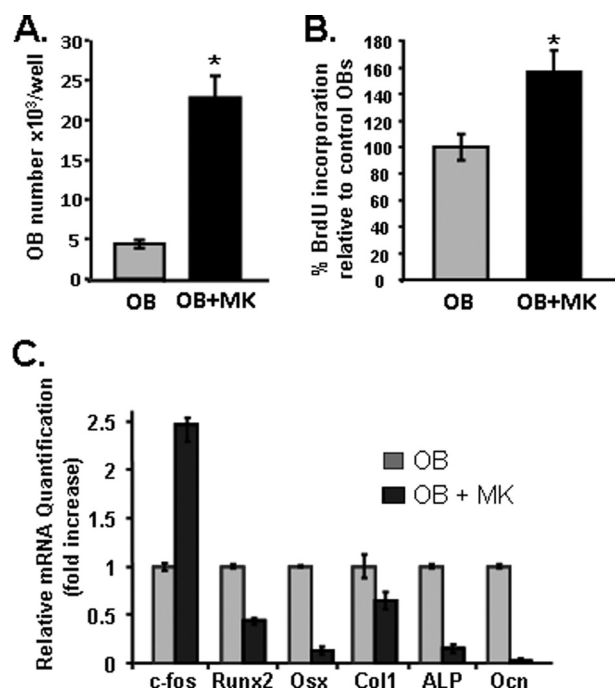
FIGURE 1. **Effects of MKs on OB number, BrdU incorporation, and expression of OB markers.** *A,* OBs, grown with or without MKs for 4 days, were counted. *B,* OBs were cultured with or without MKs for 4 days, and BrdU was added for the final 16 h of culture. MKs were removed by washing, and BrdU incorporation was measured. Results are expressed as a percentage relative to OBs cultured alone. *Asterisks* indicate a significant difference compared with OB controls, $p < 0.05$. *C,* OBs were grown with or without MKs for 4 days. MKs were removed, and OBs were analyzed by qPCR for the expression of c-*fos*, *runx2*, osterix (*Osx*), type I collagen (*Col1*), alkaline phosphatase (*ALP*), and osteocalcin (*Ocn*). For all primer pairs, results for OBs cultured with MKs were statistically different ($p < 0.05$) from OB controls.

Immunofluorescent Microscopy—Calvarial OBs were plated on glass coverslips with or without MKs and incubated for 16 h. Cells were fixed with 4% formaldehyde and washed in PBS. OBs were labeled with rhodamine phalloidin to detect actin (Sigma). Pyk2 antibodies were from BD Biosciences. Fluorescent-conjugated secondary antibodies were from Invitrogen. Cells were imaged using a Leica DMI4000 inverted microscope with attached digital camera using ImagePro 7.0 software and minimally processed with Adobe Photoshop 5.0.

Statistics—Unless otherwise stated, all data are presented as the means ± S.D. One-way analysis of variance with least significant difference multiple comparisons was used to analyze and determine statistical significance ($p < 0.05$) as appropriate. All analyses were performed with the Statistical Package for Social Sciences (IBM SPSS 19; SPSS, Inc., Co., Armonk, NY).

RESULTS

MKs Increase BrdU Incorporation and c-fos Expression in OBs—We recently reported that when OBs are co-cultured with MKs for 1–5 days, an increase in OB numbers and/or proliferation is observed (1, 2, 4, 6, 20). In this study, we further examined the effect of MKs on OB proliferation. OBs were grown in the presence or absence of MKs for 4 days. After co-culture, MKs were removed by repeated washing, and OB number was enumerated. As we reported previously, OB number was significantly increased in OBs that were cultured with MKs (Fig. 1*A*). We also examined the effect of MKs on OB

proliferation by examining BrdU incorporation in OBs. BrdU incorporation into OBs was quantified after removal of MKs (Fig. 1*B*). Consistent with the increase in OB numbers, we observed a significant increase in BrdU incorporation in OBs that had been cultured with MKs, compared with OBs cultured alone.

OB maturation requires the differentiation of pre-OBs into OBs, which then further differentiate into mature OBs. Therefore, we next sought to determine whether the newly formed OBs in our co-cultures represented immature or mature OBs. To address this, we performed qPCR of OBs (after removal of MKs) using oligonucleotide primers specific to OB transcription factors or functional proteins as follows: proliferating/pre-OB (c-*fos*), pre-OB to OB transition (Runx2, Osx), and OB/mature OBs (Col1, alkaline phosphatase, and Ocn) (21). As shown in Fig. 1*C*, we observed a significant increase in c-*fos* expression in OBs co-cultured with MKs, compared with OBs cultured alone. In contrast, *runx2* and *Osx* expressions were much lower in OBs cultured with MKs, as were all other markers of mature OBs (*Col1*, *ALP*, and *Ocn*). Together, these findings suggested that MKs stimulate the proliferation of early OBs.

Regulation of Pyk2 and FAK Levels in MK-stimulated OBs—The focal adhesion tyrosine kinase Pyk2 has been linked to the proliferation and survival of several cell types (13, 14, 22–24). We also found that OBs from mice lacking Pyk2 (Pyk2 knockout (KO)) were less responsive to the MK-induced increase in OB number than wild-type OBs (20).[4] We therefore further examined the role of Pyk2 in the signaling cascades activated by MKs in OBs. Because Pyk2 activation is an early signaling event following integrin engagement (8), we used short co-culture times for these studies (10 min to 24 h). First, we performed Western blotting to examine if MKs regulate the level of Pyk2 in wild-type OBs. OBs were cultured alone or with MKs and then MKs were removed by repeated washing prior to Western blot analysis of OBs (Fig. 2). Western blot analysis confirmed that Pyk2 was expressed in OBs cultured in the presence or absence of MKs. However, in OBs co-cultured with MKs, we observed a rapid and transient decrease in Pyk2, compared with control OBs. We confirmed equal loading of proteins in all wells by blotting for β-actin, which migrates at 40 kDa. The decrease in Pyk2 levels was observed as early as 10 min after the addition of MKs and remained low for at least 4–6 h. After 24 h, Pyk2 levels were slightly increased in MK-treated OBs, compared with OBs cultured alone (Fig. 2, *A* and *B*). We also examined Pyk2 levels in OBs co-cultured with MKs for up to 5 days but did not observe any further change in Pyk2 (data not shown).

Pyk2 is highly homologous to FAK, which also plays an essential role in cell adhesion (25) and is known to respond to mechanical strain (9, 27, 28). We examined if FAK levels in OBs were also regulated by MKs. Although FAK was readily detectable in OBs cultured alone, we were unable to detect FAK in OBs co-cultured with MKs for 1, 16, or 24 h (Fig. 2*C*) or when OBs were cultured with MKs for up to 5 days (data not shown).
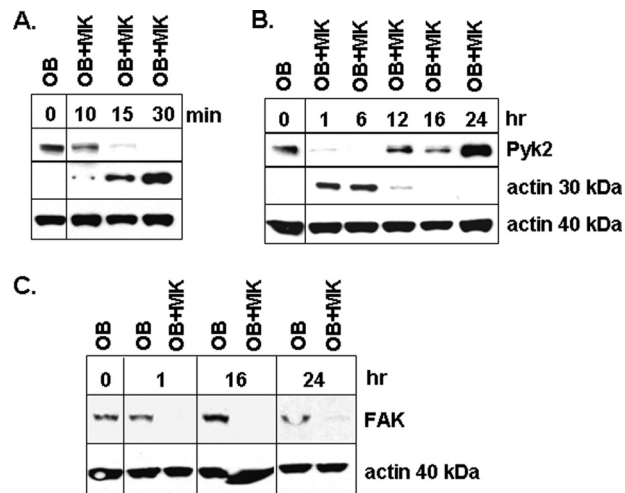
FIGURE 2. **Temporal regulation of Pyk2, FAK, and 30 kDa actin in MK-treated OBs.** *A–C,* Western blots of OB cell lysates from 2 day-old C57BL/6 mouse calvaria. OBs were serum-starved overnight, and C57BL/6 MKs were added for the indicated times and then removed by washing. OBs were lysed and resolved by SDS-PAGE. Blots were labeled with Pyk2, β-actin, or FAK.

These findings suggest that FAK and Pyk2 may have different functional roles in OBs in response to MKs.

Transient Regulation of 30 kDa of Actin in MK-stimulated OBs—Concomitant with the decrease in Pyk2 levels in OBs co-cultured with MKs, we observed the transient increase in a 30-kDa protein that was detected with our antibody to β-actin (Fig. 2*A*). The 30-kDa band was evident 10–30 min after the addition of MKs and persisted for more than 6 h, depending on our culture conditions. In contrast, full-length β-actin (40 kDa), which we used as our loading control, remained relatively constant in untreated OBs and MK-treated OBs. Mass spectrometric analysis combined with Western blotting analysis (data not shown) revealed that the 30-kDa protein detected in MK-treated OBs was likely to be a cleavage product of full-length β-actin. Interestingly, our studies revealed that Pyk2 and 30 kDa β-actin were inversely regulated by MKs, although some overlap in the expression of these proteins was observed (for example, at 1 h and 12 h) (Fig. 2, *A* and *B*). Because Pyk2 is involved in actin remodeling (8, 12), we determined if Pyk2 could potentially interact with actin in OBs. We generated full-length Pyk2 linked to glutathione *S*-transferase (GST) and performed GST-pulldown assays using lysates from OBs. Proteins were resolved by SDS-PAGE and Pyk2-interacting proteins subject to in-gel digestion and analyzed using mass spectrophotometry. These studies confirmed that in OBs, Pyk2 can potentially form a complex with β-actin (data not shown).

Because the time course for the decrease in Pyk2 levels inversely correlated with the increase in 30 kDa β-actin, we examined whether Pyk2 was necessary for the cleavage of actin. We used calvarial OBs generated from Pyk2 KO mice to examine the effect of MKs on 30 kDa β-actin in OBs. The level of full-length 40 kDa actin was unchanged by MKs in either wild-type OBs or Pyk2 KO OBs (Fig. 3*A*), confirming equal loading of proteins in each lane. However, in Pyk2 KO OBs, MKs induced the appearance of 30 kDa by 6 h, which was further increased at 12 h. Although the time course for the appearance of 30 kDa actin in Pyk2 KO OBs overlapped with that observed in wild-

A.

B.

C.
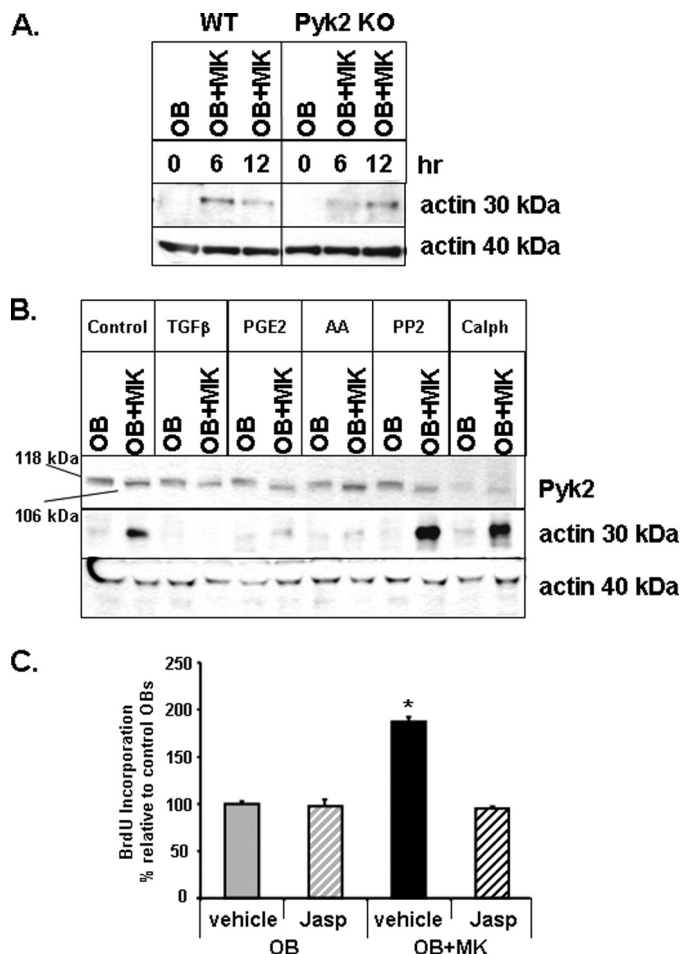
FIGURE 3. **Regulation of actin cleavage in MK-stimulated OBs.** *A,* Western blot of cell lysates from wild-type OBs or Pyk2 KO OBs cultured in the presence or absence of MKs. Blots were labeled for 30 kDa of β-actin. 40 kDa of β-actin was used as a loading control. *B,* Western blot of OBs co-cultured in the presence or absence of MKs. OBs were grown for 4 days (70% confluence), and MKs were added for 16 h and then removed by washing. OBs were incubated in media containing 1% FBS for 1 h with either transforming growth factor β (TGF-β, 2 ng/ml), prostaglandin E_2 (*PGE₂*, 100 μM), ascorbic acid (*AA*, 50 μM), PP2 (10 μM), or calphostin (*Calph*, 1 μM). The molecular mass of the two Pyk2 isoforms is indicated. *C,* OBs were cultured alone or with MKs for 4 h prior to the addition of 10 μM BrdU with or without 1 μM jasplakinolide (*Jasp*) or vehicle (*DMSO*). The cultures were continued for an additional 2 h, after which MKs were removed and OBs were analyzed for BrdU incorporation. Results are expressed as a percentage relative to control OBs (no jasplakinolide, no MKs). *Asterisk* indicates that a statistically significant difference was detected compared with OB controls ($p < 0.05$).

type OBs, 30 kDa actin was lower at 12 h than at 6 h (see also Fig. 2*B*). This suggested that the onset of 30 kDa of actin cleavage in response to MKs was delayed in Pyk2 KO OBs.

To gain a better understanding of the signaling pathways involved in regulating Pyk2 and 30-kDa actin levels, we cultured OBs in the presence or absence of MKs and then stimulated OBs with various cytokines and growth factors that promote OB differentiation. To avoid any indirect effects of the growth factors on MKs, we removed MKs by repeated washing prior to treating OBs. Purified OBs were incubated with either transforming growth factor β (TGF-β), ascorbic acid (AA), or prostaglandin E_2 (PGE₂), which are known to activate signaling pathways leading to OB differentiation (29–32). The culture conditions/timing for these studies was also chosen to enable both Pyk2 and actin to be detected in OBs. As shown in Fig. 3*B*,

Western blotting revealed very little change in total Pyk2 in control OBs or MK-treated OBs incubated with either TGF-β, AA, or PGE₂. Full-length 40-kDa actin was also unchanged by any treatment. However, TGF-β, AA, and PGE₂ significantly decreased 30-kDa actin levels in OBs previously cultured with MKs (Fig. 3*B*, *OB+MK*), suggesting that 30 kDa actin was negatively associated with OB differentiation.

Pyk2 is a substrate of the tyrosine kinase Src (12, 33) and protein kinase C (PKC) (34). We examined whether Pyk2 was regulated by inhibition of Src or PKC using the chemical inhibitors PP2 or calphostin, respectively (35, 36). OBs were cultured in the presence or absence of MKs, washed to remove MKs, and then incubated for 1 h with PP2 or calphostin (Fig. 3*B*). Although PP2 had little effect on Pyk2 levels in OBs, inhibition of PKC with calphostin significantly reduced Pyk2 in OBs, regardless of whether OBs were cultured alone or with MKs (Fig. 3*B*). In contrast, 30 kDa β-actin was increased in cells stimulated with either PP2 or calphostin. These findings suggest that the intracellular signaling pathways leading to the appearance of 30 kDa β-actin are likely to involve both Src and PKC, whereas Pyk2 levels appear to be regulated only by PKC.

Of particular note, Western blotting of Pyk2 in OBs prepared alone or in the presence MKs revealed a shift in the apparent molecular mass of Pyk2 from 118 to 106 kDa (Fig. 3*B*). The molecular weight shift of Pyk2 was unaffected by TGF-β, AA, PGE₂, or PP2, whereas calphostin decreased both species of Pyk2. As discussed below, this observation led to the discovery that MKs regulate the expression of alternatively spliced isoforms of Pyk2.

Given that MKs stimulate the proliferation of early OBs (Fig. 1) and that the MK-stimulated increase in 30 kDa β-actin was blocked by agents that promote OB differentiation (Fig. 3*B*), we asked if remodeling of the actin cytoskeleton was involved in the proliferative effects of MKs on OBs. OBs cultured in the presence or absence of MKs were treated with the actin-polymerizing and filament-stabilizing compound, jasplakinolide (37). Because 30 kDa β-actin was highest at 6 h (Fig. 2*B*), OBs were cultured with or without MKs for a total of 6 h. BrdU was added for the final 2 h of culture as was jasplakinolide or vehicle control. Cultures were washed free of MKs before BrdU incorporation in OBs was quantified (Fig. 3*C*). As expected in OBs co-cultured with MKs, we observed an increase in BrdU incorporation, compared with control OBs. Interestingly, jasplakinolide had no effect on BrdU incorporation in OBs cultured alone. However, it significantly decreased BrdU uptake into OBs co-cultured with MKs, down to the level of control OBs. A similar inhibitory effect of jasplakinolide was observed in OBs co-cultured with MKs for only 2 h (data not shown). These data suggest that actin stabilization by jasplakinolide blocks OB proliferation by MKs.

MKs Stimulate the Caspase-mediated Cleavage of Actin— The appearance of 30 kDa β-actin in OBs co-cultured with MKs is consistent with reports that actin undergoes C-terminal cleavage by the caspases, resulting in the production of several actin fragments, including a 30-kDa product (38). To examine if MKs stimulate the caspase-mediated cleavage of actin in OBs, we used a cleavable fluorescent caspase 3/7 substrate (Z-VAD-FMK) and examined changes in relative fluorometric units in
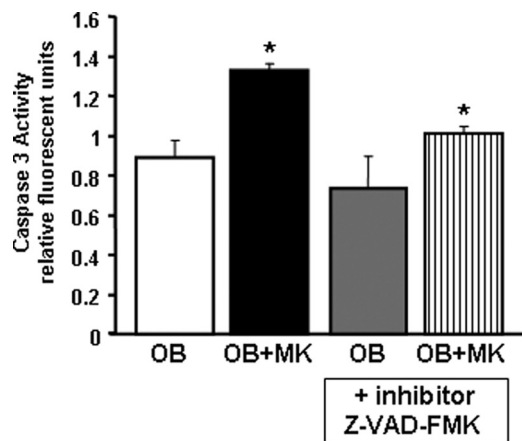
FIGURE 4. **MKs enhance caspase 3 activity in OBs.** OBs were cultured alone or in the presence of MKs for 1 h. Some cultures were treated with 20 μM Z-VAD-FMK for 16 h to inhibit caspase 3 activity. The MKs were washed off, and caspase activity in OBs was quantified by measuring fluorescence intensity. OBs cultured with MKs exhibited a significant 49% increase in caspase activity ($n = 3$ and $p = 0.01$). The addition of 20 μM Z-VAD-FMK resulted in a marked 32% reduction in caspase 3 activity in OBs co-cultured with MKs ($n = 3$ and $p = 0.0003$). *Asterisk* denotes a significant difference was detected ($p < 0.05$). Representative data from a total of three experiments are shown.

OBs before and after co-culture with MKs (MKs were removed prior to measurement) (Fig. 4). Consistent with the appearance of the 30-kDa actin at 1 h (see Fig. 2*B*), we found a 49% increase ($n = 3$ and $p = 0.01$) in relative fluorometric units in OBs co-cultured with MKs, compared with OBs cultured alone (Fig. 4). In addition, we found that OBs co-cultured with MKs for 16 h, which corresponds to the disappearance of cleaved actin, exhibited a 16% reduction ($n = 3$ and $p = 0.03$) in relative fluorometric units compared with untreated OBs (data not shown). Although caspase activity was also reduced in OBs cultured alone, the difference was not statistically significant (21% decrease, $n = 3$ and $p = 0.2$). Similar experiments were performed in the presence or absence of the caspase 3 chemical inhibitor Z-VAD-FMK (20 μM), which resulted in a 32% decrease in caspase 3/7 activity in OBs co-cultured with MKs ($n = 3$, $p < 0.001$). These findings suggest that caspase 3/7 is involved in the cleavage of β-actin in OBs pre-cultured with MKs.

Caspase-mediated actin cleavage is reported to take place prior to DNA fragmentation and apoptosis (39–41). To determine whether the caspase-mediated cleavage of actin in our cultures was associated with an increase in OB apoptosis, we performed flow cytometry and examined the levels of annexin V, an indicator of apoptosis (42). However, we did not detect any statistical difference in the number of annexin V-positive and propidium iodide-negative) cells in OBs pre-cultured with MKs for 1 or 16 h, which corresponded to the appearance and disappearance of 30 kDa actin, respectively (see Fig. 2*A*). Less than 2% of all cultures examined were annexin V-positive/propidium iodide-negative (data not shown). These findings suggested that OBs pre-cultured with MKs were unlikely to be undergoing apoptosis, although the transient activation of an apoptotic signaling pathway cannot be ruled out. The lack of apoptotic markers in our OBs was also consistent with the MK-stimulated increase in OB number, BrdU incorporation, and c-*fos* expression that we observed (Fig. 1).
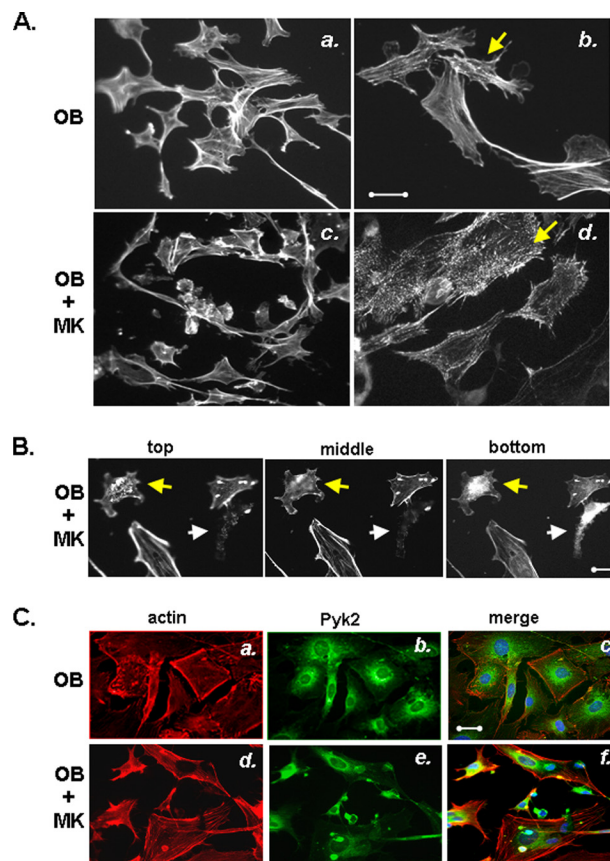
FIGURE 5. **MKs induce actin cytoskeletal re-organization in OBs.** *A,* microscopic analysis of actin in OBs cultured in the absence (*panels a* and *b*) or presence (*panels c* and *d*) of MKs for 16 h. Cells were labeled for rhodamine phalloidin to detect actin. *Yellow arrows* indicate OBs with focal adhesions. Note increased number of focal adhesions in the presence of MKs. *B,* OBs were plated as above and stained with phalloidin. Cross-sectional cell images taken through the *z*-plane were taken at the apical surface (*top*), *middle*, and at the *bottom* where OBs attach the coverslip, showing different cytoskeletal elements in these different planes. *Yellow arrows* indicate a cell that has different actin structures at the top and the bottom of the cell. *White arrows* point to a cell in which actin was only visible at the bottom *z*-plane (expressing only focal adhesions). *C,* OBs were cultured alone (*panels a–c*) or in the presence of MKs (*panels d–f*) for 6 h. Cells were stained for actin (*red, panels a* and *d*) and Pyk2 (*green, panels b* and *e*). Nuclei were stained with DAPI (*blue*). Fluorescent images were merged to show the localization of Pyk2 and actin (*merge, panels c* and *f*). Cells were imaged at $\times$40 magnification, and representative images are shown. *Scale bar,* 10 μm.

Cytoskeletal Regulation of OBs by MKs—The caspase-mediated cleavage of actin is also associated with cytoskeletal rearrangement (43). We therefore examined the effect of MKs on the morphology of OBs and the localization of Pyk2 and actin. For these studies, we either left OBs untreated or co-cultured them with MKs for 16 h and stained cells with an anti-Pyk2 antibody or with rhodamine phalloidin to detect actin. As shown in Fig. 5*A*, actin stress fibers were the predominant cytoskeletal structure present in untreated OBs. However, in a small number of OBs, focal adhesions were detected that were localized to the basolateral membrane in contact with the stratum (Fig. 5*A*, *yellow arrows*). In comparison, in OB-MK co-cultures there was an increase in the number of cells presenting focal adhesions and in the number of focal adhesions present in individual OBs (Fig. 5*A*, *panels a–c*). Although shorter time points (30 min and 1 and 6 h) were examined (data not shown), which corresponded to the appearance and disappearance of

actin, focal adhesions were most abundant after 16 h of co-culture. *z* axis scanning of cells also revealed the presence of apical actin bundles in OBs co-cultured with MKs (Fig. 5*B*). These apical actin structures were seen as early as 30 min after the addition of MKs but were not observed in untreated OBs (data not shown).

We next examined Pyk2 localization in OBs cultured in the presence or absence of MKs. In untreated OBs, Pyk2 showed a predominantly perinuclear and cytoplasmic localization (Fig. 5*C*, *panel b*). A similar localization of Pyk2 was also seen in OBs co-cultured with MKs for 6 h (Fig. 5*C*, *panel e*). In addition, co-localization of Pyk2 with cytoplasmic and perinuclear actin was seen in OBs cultured with MKs (Fig. 5*C*, *panel f, arrow*), but not in OBs cultured alone (Fig. 5*C*, *panel c*). However, we saw little co-localization of Pyk2 with actin at focal adhesions in OBs cultured in the presence or absence of MKs (Fig. 5*C*, *panels c* and *f*). These observations suggested that Pyk2 and actin may be part of overlapping, but distinct, signaling cascades induced by MKs. This is consistent with our earlier findings that MKs stimulate actin cleavage in Pyk2 KO OBs, albeit with a delayed time of onset (Fig. 3*A*).
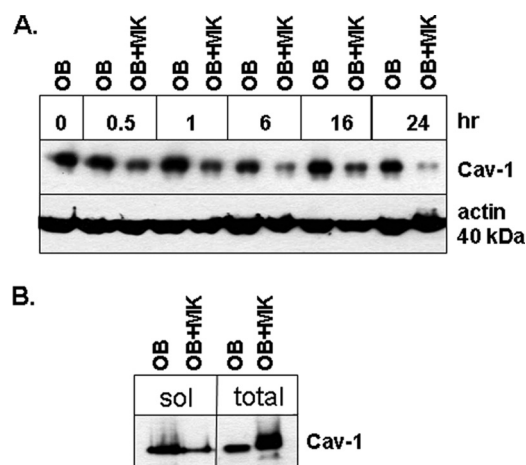
MKs Stimulate the Intracellular Translocation and Proteosome-mediated Degradation of Pyk2 in OBs—Pyk2 has been detected in different subcellular compartments, including the cytosol, nucleus, cytoskeleton, and plasma membrane. The intracellular localization of Pyk2 is regulated by different cellular conditions and by specific cytokines or integrin clustering (8, 36, 44, 46, 47). Our studies indicated that Pyk2 protein levels transiently decreased in OBs co-cultured with MKs (see Fig. 2). However, the apparent decrease in Pyk2 levels could be explained by the intracellular translocation of Pyk2 to a subcellular fraction that was not readily resolved by our cell lysis buffer (mRIPA), such as cytoskeletal or plasma membrane-bound proteins. Therefore, to examine the intracellular distribution of Pyk2 in OBs cultured in the presence or absence of MKs, we performed subcellular fractionation on OBs cultured alone or in the presence of MKs for 16 h. This time point was chosen because we found an increase in Pyk2 expression by 16 h (Fig. 2*B*). 100% of all fractions for each group were resolved by SDS-PAGE, and Western blotting was performed. Pyk2 expression was quantified by densitometry, and our findings were expressed as a percentage of total Pyk2 for each group (sum of Pyk2 in OB or OB+MK fractions) (Fig. 6*A*). In untreated OBs, ~40% of Pyk2 was detected in the cytosolic fraction, although much less Pyk2 was detected in other fractions (cytoskeletal > membrane > nuclear). In OBs co-cultured with MKs for 16 h, Pyk2 levels were also highest in the cytosolic fraction (40%), although lesser amounts of Pyk2 was detected in the other fractions (membrane > cytoskeletal > nuclear). Moreover, in MK-treated OBs, there was an increase in the relative levels of Pyk2 in the membrane fraction compared with the cytoskeletal fraction, although the percentage of Pyk2 in the nuclear fraction remained relatively unchanged in the presence or absence of MKs. Of note, Western blotting again revealed the presence of different molecular weight species of Pyk2, similar to our previous observations (see Fig. 3*B*).

Recently published studies suggest that Pyk2 undergoes proteosome-mediated degradation (48). Therefore, the transient
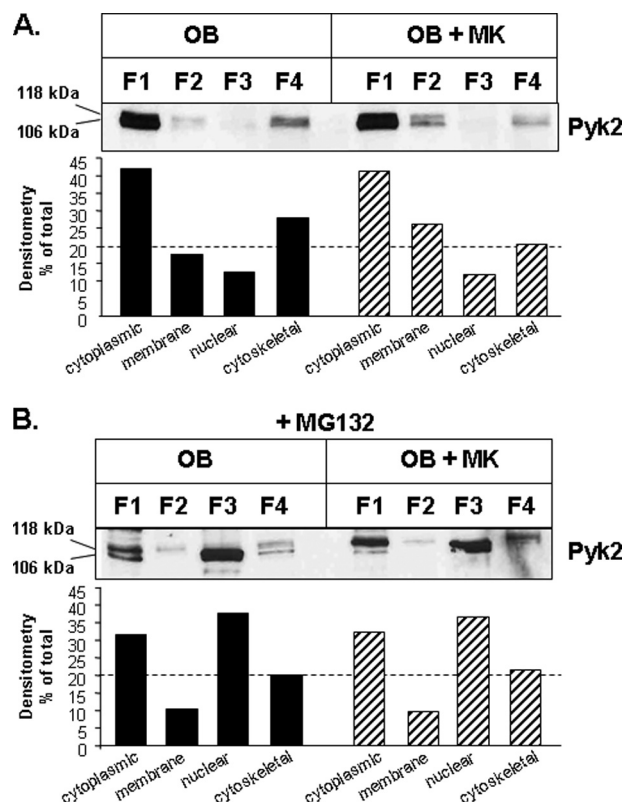
FIGURE 6. **Subcellular localization of Pyk2 in OBs is regulated by MKs.** *A*, Western blot and densitometry of subcellular fractions of OBs cultured in the presence or absence of MKs. OBs were cultured with or without MKs for 16 h. MKs were removed by repeated washing. Equal protein from treated and untreated groups was fractionated. The relative levels of Pyk2 in each of the fractions was determined by densitometry and expressed as a percentage of total Pyk2 (sum of Pyk2 from all fractions in the OB cultures or the OBs+MK co-cultures). Experiments were reproduced three times with similar densitometric findings, and a representative result is shown. *B*, Western blot and densitometry analysis of OBs cultured with or without MKs for 16 h. MG132 (20 μM) was added for the final 4 h of culture. Results are presented as percentage of Pyk2 in each fraction relative to total Pyk2 as described above. The two molecular weight isoforms of Pyk2 are indicated.

decrease in Pyk2 in OBs co-cultured with MKs could potentially be due to changes in the stability or degradation of Pyk2. To address this, we examined whether MG132, an inhibitor of the proteosome-mediated degradation pathway, affected the level and distribution of Pyk2 in OBs cultured in the presence or absence of MKs. OBs were co-cultured with and without MKs as described above. MG132 or vehicle was added for the final 4 h of culture. MKs were then removed, and we performed subcellular fractionation and densitometric analysis of Pyk2 in OBs (Fig. 6*B*). Vehicle (DMSO)-treated cells exhibited a similar distribution of Pyk2 as controls (Fig. 6*A*). Pyk2 was detected, albeit at different levels, in all subcellular fractions in MG132-treated OBs, whether or not OBs were cultured alone or in the presence of MKs. Interestingly, ~35% of total Pyk2 was detected in the nuclear fraction in MG132-treated cells, with lesser amounts detected in other fractions (nuclear > cytosolic > cytoskeletal > membrane). The percentage of Pyk2 detected in the nuclear fraction was similar when OBs were cultured in the presence or absence of MKs. However, in the absence of MG132, only 12% of Pyk2 was present in the nuclear fraction of OBs cultured with or without MKs (Fig. 6*A*). Our findings therefore suggest that Pyk2 levels in the nuclear frac-
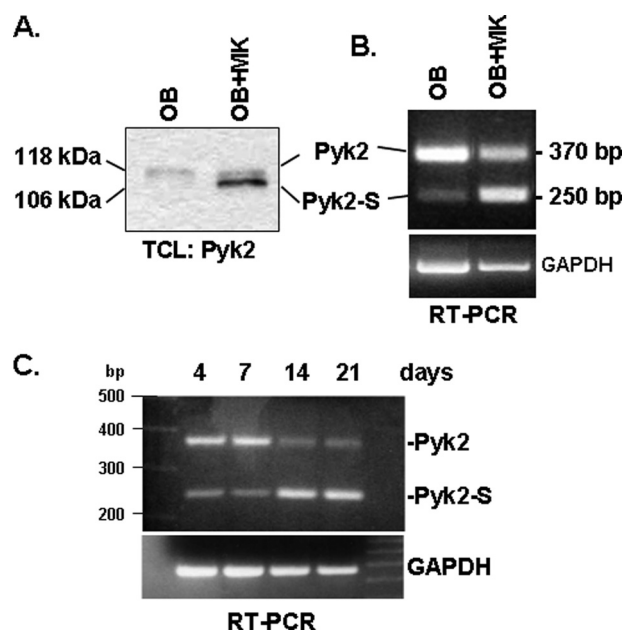
FIGURE 7. MKs induce the intracellular translocation of caveolin-1 (*Cav-1*) in OBs. *A,* Western blot showing the levels of Cav-1 in OBs cultured with or without MKs for 0.5–24 h. Cells were lysed with mRIPA buffer, and soluble proteins were resolved by SDS-PAGE. 40 kDa of actin was used as a loading control. *B,* Western blot of OBs cultured with and without MKs. Cells were lysed with mRIPA, and the soluble fraction was collected. Alternatively, cells were lysed with a 4 M urea buffer that solubilizes all proteins. Equal amounts of proteins were resolved and blotted for Cav-1.

FIGURE 8. MKs regulate the isoform-specific expression of Pyk2. *A,* Western blot of Pyk2 isoforms in OBs cultured in the presence or absence of MKs for 16 h. Note the change in the molecular mass of Pyk2 after the addition of MKs. *TCL,* total cell lysate. *B,* ethidium bromide-stained agarose gel of alternatively spliced Pyk2 isoforms. OBs were cultured with and without MKs for 16 h. GAPDH was used as a PCR control. The molecular mass of Pyk2 isoforms and the size of the expected PCR product are shown. *C,* ethidium bromide-stained gel showing changes in the relative expression of Pyk2 and Pyk2-S in OBs differentiated with osteogenic media for 4, 7, 14, and 21 days. Molecular mass standards are shown.

tion are regulated in part by proteosome-mediated degradation, independent of the presence of MKs. However, because MKs were present when MG132 was added, we cannot exclude the possibility that changes in the nuclear levels of Pyk2 could be due to changes in how MKs initially signal to OBs.

Because MKs increased the amount of Pyk2 in the membrane fraction, we examined whether other known plasma membrane-associated proteins were also altered by MKs. We used caveolin-1, a marker of the plasma membrane, which is known to be highly enriched in caveolae in the plasma membrane (49, 50). Similar to our studies shown in Fig. 2, OBs were cultured in the presence or absence of MKs for 0.5–24 h, and the level of caveolin-1 in the cytosolic fraction (prepared using mRIPA) was determined by Western blotting (Fig. 7*A*). Actin (40 kDa) was used as a loading control. These studies revealed a progressive time-dependent decrease in caveolin-1 (Cav-1) in the cytosolic fraction of OBs co-cultured with MKs, compared with OBs grown alone. To confirm that caveolin-1 was not simply being degraded over time, we cultured OBs with MKs for 16 h and then used a lysis buffer containing 4 M urea to extract total proteins in OBs. As shown in Fig. 7*B*, caveolin-1 actually increased in MK-treated OBs lysed with urea buffer (total) compared with OBs lysed with mRIPA (soluble). Therefore, these studies suggest that Pyk2 as well as caveolin-1 are translocated to the plasma membrane in the presence of MKs.

MKs Regulate the Alternative Splicing of Pyk2 Isoforms—In several of our Western blots (Figs. 2*B* and 3*B*) and subcellular fractionations (Fig. 6), we observed a shift in the molecular mass of Pyk2. The appearance of the 106-kDa Pyk2 appeared as early as 1 h after the addition of MKs to OB cultures and increased with the length of time that OBs were co-cultured with MKs (Fig. 2*B*), suggesting this was not simply an artifact of our experimental conditions. Moreover, we found that the level of 106 kDa of Pyk2 was more pronounced in OBs treated with MG132 (Fig. 6*B*). Therefore, we used SDS-polyacrylamide gels with a low percentage of acrylamide to further examine the molecular

mass forms of Pyk2 in OBs cultured with or without MKs for 16 h as in Fig. 2*B*. These studies confirmed that MKs promote the appearance of two molecular mass species of Pyk2 of 118 and 106 kDa (Fig. 8*A*). Darker exposures of blots also showed the presence of comparatively low levels of 106 kDa of Pyk2 in OBs cultured in the absence of MKs (data not shown).

Several distinct molecular mass isoforms of Pyk2 have been reported in different tissues, which are produced by alternative mRNA splicing of *Pyk2* mRNA (51). A 106-kDa Pyk2 isoform has been reported that lacks a 42-amino acid insert found in full-length Pyk2 (118 kDa) (51). Given the apparent molecular weight of Pyk2 in our MK-treated OBs, we predicted these proteins were likely to represent full-length Pyk2 and the shorter splice variant of Pyk2, which we named Pyk2-S. To confirm the presence of alternatively spliced *Pyk2* mRNA in OBs, we performed reverse transcription-PCR using oligonucleotide primers spanning the known exon splice site. Indeed, as shown in Fig. 8*B*, OBs express full-length Pyk2 (corresponding to the 370-bp PCR product) and the shorter splice variant (250-bp PCR product), which likely encodes the 106-kDa isoform Pyk2-S. These studies also revealed that full-length Pyk2 (370 bp) was more highly expressed in untreated OBs than Pyk2-S (250 bp), whereas in MK-treated OBs, Pyk2-S expression was higher than full-length Pyk2 (Fig. 8*B*). These findings were consistent with our Western blot results (see Figs. 2*B*, 3*B*, and 8*A*).

To begin to investigate the functional role of Pyk2 and Pyk2-S in OBs, we examined whether the expression of Pyk2 and Pyk2-S mRNA was regulated during normal OB differentiation. Calvarial OBs were cultured under osteogenic conditions

(differentiation media) containing ascorbic acid and β-glycerol phosphate for up to 21 days. RNA was reverse-transcribed, and RT-PCR was performed using primer pairs spanning the isoform mRNA splice site (Fig. 8C). Similar to our findings with OBs co-cultured with or without MKs, we observed two Pyk2 mRNA species in OBs cultured in osteogenic media for 4–21 days. In addition, these studies revealed a decrease in the ratio of Pyk2 to Pyk2-S during OB differentiation, with Pyk2-S expression exceeding Pyk2 by day 14 and day 21. The change in the relative expression of Pyk2 and Pyk2-S during OB differentiation potentially suggests that the Pyk2 isoforms are potentially important for OB function, although this remains to be determined.

DISCUSSION

The nonreceptor kinase Pyk2 plays a role in many diverse cellular activities, including proliferation, migration, and function of mesenchymal, epithelial, and hematopoietic cells (7–10). Deletion of the *Pyk2* gene in mice leads to an increase in bone mass that is due to defects in the activity of both OBs (7) and osteoclasts (11). OBs differentiated from Pyk2 KO mice exhibit an increase in alkaline phosphatase activity and OB mineralization (7), although selective Pyk2 chemical inhibitors block Pyk2 auto-phosphorylation and increase osteogenic markers in OB-like cells (52). In addition, osteogenesis is increased in mice with OB-targeted deletion of the Pyk2 homolog FAK (53). Our recent studies demonstrate that Pyk2 KO OBs are less responsive to the stimulatory effect of MKs on OB numbers (20)[4] supporting a role for Pyk2 in MK-induced signaling cascades leading to OB proliferation. In agreement of these findings, we now report that MKs increase BrdU incorporation in OBs and stimulate the expression of c-*fos* mRNA, a transcriptional marker of proliferating OBs (21). Interestingly, elevated levels of c-*fos* in OBs have also been associated with accelerated S phase entry due to deregulated activity of cyclinA and E-CDK2 (54). In others studies, we also observed a reduction in the levels of p53, a cell cycle arrest protein, in OBs co-cultured with MKs (20).[4] It was also recently reported that Pyk2 regulates proliferation and survival of fibroblasts and ovarian carcinoma cells by promoting p53 degradation (13, 14). Thus, we predict that cell cycle entry and/or progression in OBs co-cultured with MKs is likely regulated by Pyk2 and p53, although this remains to be determined.

Cleavage of actin at aspartate 244 by cysteinyl aspartate-specific proteases (caspases) generates an N-terminal 32-kDa and C-terminal 15-kDa actin fragment. We found that MKs increased 30 kDa actin in OBs, which was likely to be the result of caspase-mediated actin cleavage. Although the cleavage of actin by the caspases has been linked with cellular apoptosis, the lack of apoptotic markers in our OB+MK co-cultures as well as the increase in OB numbers, BrdU incorporation, and c-*fos* expression strongly argue against the induction of apoptosis in OBs co-cultured with MKs, although the transient activation of the apoptotic pathway cannot be ruled out. Instead, our findings support a role for caspase-mediated actin cleavage in cytoskeletal remodeling as reported by others (43). This is consistent with reports that caspase 3 activation can occur, in the absence of cell death, in pre-osteoblastic MC3T3 cells (55).

Along similar lines, we found that 30 kDa β-actin was downregulated in the presence of TGF-β, PGE$_2$, and ascorbic acid, which are known inducers of OB differentiation (Fig. 3B). These findings suggested that actin cleavage may also be associated with MK-regulated signaling pathways leading to OB proliferation. Indeed, we found that treatment of OB-MK co-cultures with jasplakinolide, which stabilizes actin filaments (37), suppressed the stimulatory effect of MKs on BrdU incorporation and restored BrdU to control levels (OBs cultured without MKs) (Fig. 3C). Of further interest, actin filaments are also important components of the process leading to the assembly, trafficking, and stabilization of gap junctions, and we previously found that MKs can communicate to OBs through gap junction intracellular communication (4).

In this study, we also examined whether Pyk2 and FAK were involved in the signaling cascades activated by MKs in OBs. Our studies demonstrated that MKs regulate the rapid and transient decrease of Pyk2, coincident with a transient increase in the cleavage of β-actin. We also found by mass spectrophotometry that Pyk2 could form a complex with β-actin (data not shown) and was in part co-localized with actin in OBs co-cultured with MKs (Fig. 5). However, because actin cleavage was evident, albeit temporally delayed, in Pyk2 KO OBs co-cultured with MKs (Fig. 3A), it is likely that Pyk2 and actin share overlapping, but distinct, signaling pathways in response to MKs. Similarly, because Pyk2 levels were transiently decreased, whereas FAK exhibited a sustained decrease in response to MKs, it is likely that these focal adhesion tyrosine kinases have distinct roles in OBs in response to MKs. Our studies also revealed a dramatic change in the morphology of OBs following the addition of MKs, resulting in cell spreading and an increase in the appearance of focal adhesions (Fig. 5). The increased appearance of focal adhesions in OBs co-cultured with MKs is also consistent with reports that FAK KO OBs have increased numbers of focal adhesions and exhibit decreased migration (56, 57). It is therefore possible that the increase in focal adhesions occurs as a result of the sustained decrease in FAK levels in OBs co-cultured with MKs. We also observed two populations of actin present on the polar surfaces of OBs co-cultured with MKs (Fig. 5B). Although cell-cell communication was not a focus of this study, we speculate that the apical actin bundles may be involved in MK-OB communication, similar to what was reported in Madin-Darby canine kidney (MDCK) cells (58). Interestingly, the apical population of actin described in MDCK cells was associated with zonula adherens. Given that MK-OB signaling occurs in part via gap junctions (4), it will be of interest to determine the role of actin in zonula adherens and/or gap junctions in OB-MK communication.

Several studies published by others reveal that Pyk2 undergoes intracellular translocation in response to integrins and cytokines (8, 46, 59, 60). The translocation of Pyk2 to the nucleus has also been reported (59, 61). Our biochemical studies further support these findings. We found that Pyk2 was expressed in several intracellular compartments of OBs and that MKs induced the partial translocation of Pyk2 from the cytoskeletal fraction to the plasma membrane. We also found that 10–15% of Pyk2 was localized to the nucleus in OBs and that MG132, which blocks proteosome-mediated degradation,

increased the level of nuclear Pyk2 to ~35% of total Pyk2. These novel findings indicate that nuclear Pyk2 is degraded by the proteosome-ubiquitin pathway and that this occurs independently of MK-activated signaling in OBs. Given that MKs induced the translocation of caveolin-1 to the plasma membrane, it is possible that Pyk2 perhaps participates in caveolin-1-mediated internalization of activated integrins and cytokine receptors or in cell-cell communication via gap junctions, which has also been shown to involve caveolin-1 (62–65).

Alternative mRNA splicing of the *Pyk2* gene results in the production of a 106-kDa Pyk2 isoform, designated Pyk2-S, which lacks a 42-amino acid insert found in full-length Pyk2 (118 kDa) (51, 51, 66, 67). The Pyk2 isoforms have been reported in a number of tissues and cell lines such as brain, spleen, and B cells, as well as other hematopoietically derived cells. However, the functional role of these proteins is unknown. We found that MKs increase the ratio of Pyk2-S/Pyk2 mRNA as well as increase Pyk2-S protein levels in OBs (Fig. 8). Furthermore, subcellular fractionation in the presence of MG132 indicated that Pyk2-S is potentially degraded by the proteosome pathway, whereas full-length Pyk2, which was mostly detected in the membrane and cytoskeletal fractions (Fig. 6*B*), is more resistant to degradation. Because MKs increase BrdU incorporation and c-*fos* expression in OBs, it is tempting to speculate that nuclear Pyk2-S potentially plays a role in OB proliferation in response to MKs. In contradistinction, we found that the ratio of Pyk2-S/Pyk2 also increased in OBs cultured with osteogenic media, which induces differentiation (Fig. 8*C*). Although the protein levels of Pyk2 and Pyk2-S in OBs cultured with osteogenic media remain to be determined, the decrease in Pyk2 mRNA (full-length isoform) during OB differentiation is consistent with the increased differentiation observed in Pyk2 KO OBs, compared with wild-type OBs (7). However, it is likely that several factors, including mRNA expression, subcellular localization, protein stability, kinase activity, and the potential association of Pyk2 and Pyk2-S with distinct binding partners, may be involved. Additional studies using cDNA for the individual isoforms will be necessary to elucidate the role of Pyk2 and Pyk2-S in the transition of OBs from proliferation to differentiation, and their role in MK-mediated changes in OB function. Nevertheless, our data suggest that the Pyk2 isoforms are likely to exhibit unique and possibly overlapping functions in OBs.

In summary, our studies demonstrate that MKs increase OB proliferation via signaling pathways that regulate the rapid and transient down-regulation of Pyk2, coincident with a transient increase in the caspase-mediated cleavage of actin. In addition, MKs promote the sustained down-regulation of FAK in OBs. MKs also regulate the transcription of Pyk2 isoforms, as well as the intracellular localization and proteosome-mediated degradation of Pyk2 and Pyk2-S. We report that MKs stimulate changes in OB morphology, resulting in an increase in focal adhesion formation, and that actin remodeling is likely to be important for OB-MK signaling events leading to OB proliferation. Together, the MK-mediated changes in the expression, localization, and activity of Pyk2 isoforms are likely to play a key role in the regulation of OB function and in the MK-induced increase in OB proliferation. The increased proliferative effect

of MKs on OBs is likely to be physiologically important in regulating both skeletal homeostasis (6) and hematopoiesis (26), during which MKs are intimately associated with OB lineage cells. For example, MKs can stimulate a 2–3-fold increase in OB number following total body irradiation, which in turn enhances hematopoietic stem cell recovery (45). Novel mechanisms to induce OB proliferation are therefore important for anabolic treatments to prevent or replace bone loss. Our findings represent a significant advancement in our understanding of the signaling pathways involved in regulating OB proliferation as well as proliferation in other cell types in which Pyk2 and Pyk2-S are expressed.

REFERENCES

1. Bethel, M., Srour, E. F., and Kacena, M. A. (2011) Hematopoietic cell regulation of osteoblast proliferation and differentiation. *Curr. Osteoporos. Rep.* **9,** 96–102
2. Ciovacco, W. A., Cheng, Y. H., Horowitz, M. C., and Kacena, M. A. (2010) Immature and mature megakaryocytes enhance osteoblast proliferation and inhibit osteoclast formation. *J. Cell. Biochem.* **109,** 774–781
3. Kacena, M. A., Gundberg, C. M., and Horowitz, M. C. (2006) A reciprocal regulatory interaction between megakaryocytes, bone cells, and hematopoietic stem cells. *Bone* **39,** 978–984
4. Ciovacco, W. A., Goldberg, C. G., Taylor, A. F., Lemieux, J. M., Horowitz, M. C., Donahue, H. J., and Kacena, M. A. (2009) The role of gap junctions in megakaryocyte-mediated osteoblast proliferation and differentiation. *Bone* **44,** 80–86
5. Lemieux, J. M., Horowitz, M. C., and Kacena, M. A. (2010) Involvement of integrins $\alpha_3\beta_1$ and $\alpha_5\beta_1$ and glycoprotein IIb in megakaryocyte-induced osteoblast proliferation. *J. Cell. Biochem.* **109,** 927–932
6. Kacena, M. A., Shivdasani, R. A., Wilson, K., Xi, Y., Troiano, N., Nazarian, A., Gundberg, C. M., Bouxsein, M. L., Lorenzo, J. A., and Horowitz, M. C. (2004) Megakaryocyte-osteoblast interaction revealed in mice deficient in transcription factors GATA-1 and NF-E2. *J. Bone Miner. Res.* **19,** 652–660
7. Buckbinder, L., Crawford, D. T., Qi, H., Ke, H. Z., Olson, L. M., Long, K. R., Bonnette, P. C., Baumann, A. P., Hambor, J. E., Grasser, W. A., 3rd, Pan, L. C., Owen, T. A., Luzzio, M. J., Hulford, C. A., Gebhard, D. F., Paralkar, V. M., Simmons, H. A., Kath, J. C., Roberts, W. G., Smock, S. L., Guzman-Perez, A., Brown, T. A., and Li, M. (2007) Proline-rich tyrosine kinase 2 regulates osteoprogenitor cells and bone formation and offers an anabolic treatment approach for osteoporosis. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 10619–10624
8. Duong, L. T., Lakkakorpi, P. T., Nakamura, I., Machwate, M., Nagy, R. M., and Rodan, G. A. (1998) PYK2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of $\alpha_v\beta_3$ integrin, and phosphorylated by Src kinase. *J. Clin. Invest.* **102,** 881–892
9. Boutahar, N., Guignandon, A., Vico, L., and Lafage-Proust, M. H. (2004) Mechanical strain on osteoblasts activates autophosphorylation of focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. *J. Biol. Chem.* **279,** 30588–30599
10. Xiong, W. C., and Feng, X. (2003) PYK2 and FAK in osteoclasts. *Front. Biosci.* **8,** d1219-d1226
11. Gil-Henn, H., Destaing, O., Sims, N. A., Aoki, K., Alles, N., Neff, L., Sanjay, A., Bruzzaniti, A., De Camilli, P., Baron, R., and Schlessinger, J. (2007) Defective microtubule-dependent podosome organization in osteoclasts leads to increased bone density in Pyk2$^{-/-}$ mice. *J. Cell Biol.* **178,**

1053–1064

12. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C., and Baron, R. (2001) Cbl associates with Pyk2 and Src to regulate Src kinase activity, $\alpha_v\beta_3$ integrin-mediated signaling, cell adhesion, and osteoclast motility. *J. Cell Biol.* **152,** 181–195

13. Lim, S. T., Chen, X. L., Lim, Y., Hanson, D. A., Vo, T. T., Howerton, K., Larocque, N., Fisher, S. J., Schlaepfer, D. D., and Ilic, D. (2008) Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Mol. Cell* **29,** 9–22

14. Lim, S. T., Miller, N. L., Nam, J. O., Chen, X. L., Lim, Y., and Schlaepfer, D. D. (2010) Pyk2 inhibition of p53 as an adaptive and intrinsic mechanism facilitating cell proliferation and survival. *J. Biol. Chem.* **285,** 1743–1753

15. Horowitz, M. C., Fields, A., DeMeo, D., Qian, H. Y., Bothwell, A. L., and Trepman, E. (1994) Expression and regulation of Ly-6 differentiation antigens by murine osteoblasts. *Endocrinology* **135,** 1032–1043

16. Wong, G. L., and Cohn, D. V. (1975) Target cells in bone for parathormone and calcitonin are different. Enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 3167–3171

17. Simmons, D. J., Kent, G. N., Jilka, R. L., Scott, D. M., Fallon, M., and Cohn, D. V. (1982) Formation of bone by isolated, cultured osteoblasts in Millipore diffusion chambers. *Calcif. Tissue Int.* **34,** 291–294

18. Jilka, R. L., and Cohn, D. V. (1981) Role of phosphodiesterase in the parathormone-stimulated adenosine 3′,5′-monophosphate response in bone cell populations enriched in osteoclasts and osteoblasts. *Endocrinology* **109,** 743–747

19. Kacena, M. A., Nelson, T., Clough, M. E., Lee, S. K., Lorenzo, J. A., Gundberg, C. M., and Horowitz, M. C. (2006) Megakaryocyte-mediated inhibition of osteoclast development. *Bone* **39,** 991–999

20. Bruzzaniti, A., Cheng, Y. H., Huang, S., Nguyen, K., Chitteti, B. R., Srour, E. F., Mayo, L. D., and Kacena, M. (2010) Pyk2 regulates megakaryocyte-induced osteoblast proliferation *in vitro* and bone formation *in vivo*. *J. Bone Miner. Res.* **25,** Suppl. 1, SU0248

21. Schinke, T., and Karsenty, G. (2008) in *Principals of Bone Biology* (Bilezikian, J. P., Raisz, L. G., and Martin, T. J., eds) pp. 109–119, Elsevier Inc., San Diego

22. Perez, J., Torres, R. A., Rocic, P., Cismowski, M. J., Weber, D. S., Darley-Usmar, V. M., and Lucchesi, P. A. (2011) PYK2 signaling is required for PDGF-dependent vascular smooth muscle cell proliferation. *Am. J. Physiol. Cell Physiol.* **301,** C242–C251

23. Kisslinger, A., Cantile, M., Sparaneo, G., Vitale, N., Fabbrocini, G., Chieffi, P., Cillo, C., Mancini, F. P., and Tramontano, D. (2009) cAMP and Pyk2 interact to regulate prostate cell proliferation and function. *Cancer Biol. Ther.* **8,** 236–242

24. Sun, C. K., Man, K., Ng, K. T., Ho, J. W., Lim, Z. X., Cheng, Q., Lo, C. M., Poon, R. T., and Fan, S. T. (2008) Proline-rich tyrosine kinase 2 (Pyk2) promotes proliferation and invasiveness of hepatocellular carcinoma cells through c-Src/ERK activation. *Carcinogenesis* **29,** 2096–2105

25. Richardson, A., and Parsons, T. (1996) A mechanism for regulation of the adhesion-associated protein-tyrosine kinase pp125[FAK]. *Nature* **380,** 538–540

26. Kacena, M. A., Gundberg, C. M., Nelson, T., and Horowitz, M. C. (2005) Loss of the transcription factor p45 NF-E2 results in a developmental arrest of megakaryocyte differentiation and the onset of a high bone mass phenotype. *Bone* **36,** 215–223

27. Guignandon, A., Boutahar, N., Rattner, A., Vico, L., and Lafage-Proust, M. H. (2006) Cyclic strain promote shuttling of PYK2/Hic-5 complex from focal contacts in osteoblast-like cells. *Biochem. Biophys. Res. Commun.* **343,** 407–414

28. Wozniak, M., Fausto, A., Carron, C. P., Meyer, D. M., and Hruska, K. A. (2000) Mechanically strained cells of the osteoblast lineage organize their extracellular matrix through unique sites of $\alpha_v\beta_3$-integrin expression. *J. Bone Miner. Res.* **15,** 1731–1745

29. Conconi, M. T., Tommasini, M., Baiguera, S., De Coppi, P., Parnigotto, P. P., and Nussdorfer, G. G. (2002) Effects of prostaglandins E1 and E2 on the growth and differentiation of osteoblast-like cells cultured in vitro. *Int. J. Mol. Med.* **10,** 451–456

30. van der Zande, M., Walboomers, X. F., Briest, A., Springer, M., Alava, J. I., and Jansen, J. A. (2008) The effect of combined application of TGFβ-1, BMP-2, and COLLOSS E on the development of bone marrow-derived osteoblast-like cells in vitro. *J. Biomed. Mater. Res. A* **86,** 788–795

31. Wu, X., Zeng, L. H., Taniguchi, T., and Xie, Q. M. (2007) Activation of PKA and phosphorylation of sodium-dependent vitamin C transporter 2 by prostaglandin E2 promote osteoblast-like differentiation in MC3T3-E1 cells. *Cell Death Differ.* **14,** 1792–1801

32. Gerstenfeld, L. C., Chipman, S. D., Glowacki, J., and Lian, J. B. (1987) Expression of differentiated function by mineralizing cultures of chicken osteoblasts. *Dev. Biol.* **122,** 49–60

33. Lakkakorpi, P. T., Bett, A. J., Lipfert, L., Rodan, G. A., and Duong le, T. (2003) PYK2 autophosphorylation, but not kinase activity, is necessary for adhesion-induced association with c-Src, osteoclast spreading, and bone resorption. *J. Biol. Chem.* **278,** 11502–11512

34. Bayer, A. L., Heidkamp, M. C., Howes, A. L., Heller Brown, J., Byron, K. L., and Samarel, A. M. (2003) Protein kinase C ϵ-dependent activation of proline-rich tyrosine kinase 2 in neonatal rat ventricular myocytes. *J. Mol. Cell. Cardiol.* **35,** 1121–1133

35. Emkey, R., and Kahn, C. R. (1997) Cross-talk between phorbol ester-mediated signaling and tyrosine kinase proto-oncogenes. II. Comparison of phorbol ester and sphingomyelinase-induced phosphorylation of ErbB2 and ErbB3. *J. Biol. Chem.* **272,** 31182–31189

36. Ohmori, T., Yatomi, Y., Asazuma, N., Satoh, K., and Ozaki, Y. (2000) Involvement of proline-rich tyrosine kinase 2 in platelet activation. Tyrosine phosphorylation mostly dependent on αIIbβ3 integrin and protein kinase C, translocation to the cytoskeleton, and association with Shc through Grb2. *Biochem. J.* **347,** 561–569

37. Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L., and Korn, E. D. (1994) Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269,** 14869–14871

38. Mashima, T., Naito, M., Fujita, N., Noguchi, K., and Tsuruo, T. (1995) Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. *Biochem. Biophys. Res. Commun.* **217,** 1185–1192

39. Mashima, T., Naito, M., and Tsuruo, T. (1999) Caspase-mediated cleavage of cytoskeletal actin plays a positive role in the process of morphological apoptosis. *Oncogene* **18,** 2423–2430

40. Depraetere, V., and Golstein, P. (1998) Dismantling in cell death. Molecular mechanisms and relationship to caspase activation. *Scand. J. Immunol.* **47,** 523–531

41. Cohen, G. M. (1997) Caspases. The executioners of apoptosis. *Biochem. J.* **326,** 1–16

42. Jetmore, A., Plett, P. A., Tong, X., Wolber, F. M., Breese, R., Abonour, R., Orschell-Traycoff, C. M., and Srour, E. F. (2002) Homing efficiency, cell cycle kinetics, and survival of quiescent and cycling human CD34^{+} cells transplanted into conditioned NOD/SCID recipients. *Blood* **99,** 1585–1593

43. Rohn, T. T., Cusack, S. M., Kessinger, S. R., and Oxford, J. T. (2004) Caspase activation independent of cell death is required for proper cell dispersal and correct morphology in PC12 cells. *Exp. Cell Res.* **295,** 215–225

44. Blaschke, F., Stawowy, P., Kappert, K., Goetze, S., Kintscher, U., Wollert-Wulf, B., Fleck, E., and Graf, K. (2002) Angiotensin II-augmented migration of VSMCs toward PDGF-BB involves Pyk2 and ERK 1/2 activation. *Basic Res. Cardiol.* **97,** 334–342

45. Dominici, M., Rasini, V., Bussolari, R., Chen, X., Hofmann, T. J., Spano, C., Bernabei, D., Veronesi, E., Bertoni, F., Paolucci, P., Conte, P., and Horwitz, E. M. (2009) Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. *Blood* **114,** 2333–2343

46. Litvak, V., Tian, D., Shaul, Y. D., and Lev, S. (2000) Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. *J. Biol. Chem.* **275,** 32736–32746

47. Haglund, K., Ivankovic-Dikic, I., Shimokawa, N., Kruh, G. D., and Dikic, I. (2004) Recruitment of Pyk2 and Cbl to lipid rafts mediates signals impor-

tant for actin reorganization in growing neurites. *J. Cell Sci.* **117,** 2557–2568

48. Lee, S. H., Yun, S., Piao, Z. H., Jeong, M., Kim, D. O., Jung, H., Lee, J., Kim, M. J., Kim, M. S., Chung, J. W., Kim, T. D., Yoon, S. R., Greenberg, P. D., and Choi, I. (2010) Suppressor of cytokine signaling 2 regulates IL-15-primed human NK cell function via control of phosphorylated Pyk2. *J. Immunol.* **185,** 917–928

49. del Pozo, M. A., Balasubramanian, N., Alderson, N. B., Kiosses, W. B., Grande-García, A., Anderson, R. G., and Schwartz, M. A. (2005) Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. *Nat. Cell Biol.* **7,** 901–908

50. Bartholomew, J. N., Volonte, D., and Galbiati, F. (2009) Caveolin-1 regulates the antagonistic pleiotropic properties of cellular senescence through a novel Mdm2/p53-mediated pathway. *Cancer Res.* **69,** 2878–2886

51. Xiong, W. C., Macklem, M., and Parsons, J. T. (1998) Expression and characterization of splice variants of PYK2, a focal adhesion kinase-related protein. *J. Cell Sci.* **111,** 1981–1991

52. Han, S., Mistry, A., Chang, J. S., Cunningham, D., Griffor, M., Bonnette, P. C., Wang, H., Chrunyk, B. A., Aspnes, G. E., Walker, D. P., Brosius, A. D., and Buckbinder, L. (2009) Structural characterization of proline-rich tyrosine kinase 2 (PYK2) reveals a unique (DFG-out) conformation and enables inhibitor design. *J. Biol. Chem.* **284,** 13193–13201

53. Kim, J. B., Leucht, P., Luppen, C. A., Park, Y. J., Beggs, H. E., Damsky, C. H., and Helms, J. A. (2007) Reconciling the roles of FAK in osteoblast differentiation, osteoclast remodeling, and bone regeneration. *Bone* **41,** 39–51

54. Sunters, A., Thomas, D. P., Yeudall, W. A., and Grigoriadis, A. E. (2004) Accelerated cell cycle progression in osteoblasts overexpressing the c-*fos* proto-oncogene. Induction of cyclin A and enhanced CDK2 activity. *J. Biol. Chem.* **279,** 9882–9891

55. Mogi, M., Ozeki, N., Nakamura, H., and Togari, A. (2004) Dual roles for NF-κB activation in osteoblastic cells by serum deprivation. Osteoblastic apoptosis and cell cycle arrest. *Bone* **35,** 507–516

56. Klemm, A. H., Kienle, S., Rheinlaender, J., Schäffer, T. E., and Goldmann, W. H. (2010) The influence of Pyk2 on the mechanical properties in fibroblasts. *Biochem. Biophys. Res. Commun.* **393,** 694–697

57. Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D., and Schwartz, M. A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J. Cell Sci.* **113,** 3673–3678

58. Stevenson, B. R., and Begg, D. A. (1994) Concentration-dependent effects of cytochalasin D on tight junctions and actin filaments in MDCK epithelial cells. *J. Cell Sci.* **107,** 367–375

59. Aoto, H., Sasaki, H., Ishino, M., and Sasaki, T. (2002) Nuclear translocation of cell adhesion kinase β/proline-rich tyrosine kinase 2. *Cell Struct. Funct.* **27,** 47–61

60. Sancho, D., Montoya, M. C., Monjas, A., Gordón-Alonso, M., Katagiri, T., Gil, D., Tejedor, R., Alarcón, B., and Sánchez-Madrid, F. (2002) TCR engagement induces proline-rich tyrosine kinase-2 (Pyk2) translocation to the T cell-APC interface independently of Pyk2 activity and in an immunoreceptor tyrosine-based activation motif-mediated fashion. *J. Immunol.* **169,** 292–300

61. Faure, C., Corvol, J. C., Toutant, M., Valjent, E., Hvalby, O., Jensen, V., El Messari, S., Corsi, J. M., Kadaré, G., and Girault, J. A. (2007) Calcineurin is essential for depolarization-induced nuclear translocation and tyrosine phosphorylation of PYK2 in neurons. *J. Cell Sci.* **120,** 3034–3044

62. Schubert, A. L., Schubert, W., Spray, D. C., and Lisanti, M. P. (2002) Connexin family members target to lipid raft domains and interact with caveolin-1. *Biochemistry* **41,** 5754–5764

63. Langlois, S., Cowan, K. N., Shao, Q., Cowan, B. J., and Laird, D. W. (2008) Caveolin-1 and -2 interact with connexin43 and regulate gap junctional intercellular communication in keratinocytes. *Mol. Biol. Cell* **19,** 912–928

64. Laing, J. G., Manley-Markowski, R. N., Koval, M., Civitelli, R., and Steinberg, T. H. (2001) Connexin45 interacts with zonula occludens-1 and connexin43 in osteoblastic cells. *J. Biol. Chem.* **276,** 23051–23055

65. Laing, J. G., Manley-Markowski, R. N., Koval, M., Civitelli, R., and Steinberg, T. H. (2001) Connexin45 interacts with zonula occludens-1 in osteoblastic cells. *Cell Commun. Adhes.* **8,** 209–212

66. Dikic, I., Dikic, I., and Schlessinger, J. (1998) Identification of a new Pyk2 isoform implicated in chemokine and antigen receptor signaling. *J. Biol. Chem.* **273,** 14301–14308

67. Keogh, R. J., Houliston, R. A., and Wheeler-Jones, C. P. (2002) Human endothelial Pyk2 is expressed in two isoforms and associates with paxillin and p130Cas. *Biochem. Biophys. Res. Commun.* **290,** 1470–1477